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TITLE: Investigating the Role of Akt1 in Prostate Cancer Development through Phosphorylation-dependent Regulation of Skp2 Ubiquity and Oncogenic Function

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<p>14. ABSTRACT Elevated Skp2 expression is frequently observed in many tumors including breast and prostate carcinomas. However, the molecular mechanisms underlying elevated Skp2 expression in prostate cancers have not been fully explored. Hyperactivation of the Akt pathway is considered a hallmark of many cancers and it has been reported that activation of the PI3K/Akt pathway enhances p27 destruction. Thus, we hypothesize that sustained Akt activity in prostate cancer cells leads to elevated phosphorylation of Skp2, and subsequently influences Skp2 stability and its oncogenic functions. In support of our hypothesis, we found that Skp2 abundance is affected by manipulation of the PTEN/PI3K/Akt pathway in the PC3 and LNCaP prostate cancer cell lines. Furthermore, we showed that this is partially through phosphorylation of Skp2 by Akt, which impairs Skp2 destruction by Cdh1. Sequence analysis revealed that the Akt phosphorylation site (Ser72) is localized in a putative Nuclear Localization Sequence (NLS). Consistent with this notion, we found that overexpression of Myr-Akt promoted Skp2 cytoplasmic translocation, and that inhibition of PI3K/Akt activity enhanced its nuclear localization. We believe that these studies will provide a novel mechanism for Skp2 overexpression in prostate cancers and provide the rationale for developing Akt1-specific inhibitors to treat prostate cancer patients.</p>				
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Introduction:

Project Title: Investigating the role of Akt1 in prostate cancer development through phosphorylation-dependent regulation of Skp2 stability and oncogenic function

Defective cell cycle regulation leads to genomic instability, which ultimately contributes to cancer development (3, 9). Two related, multi-subunit E3 ubiquitin ligase enzymes, the Anaphase Promoting Complex (APC) and the Skp1-Cullin1-F-box (SCF) complex are the major driving forces that govern ordered cell cycle progression through promoting the destruction of the key cell cycle regulators (8). Elevated Skp2 expression is frequently observed in many tumors including breast and prostate carcinomas (5, 11). It has been proposed that enhanced Skp2 expression leads to the accelerated degradation of targets such as p27 (4, 12) and other cell cycle regulators including FOXO1 and Orc1, thus promoting cell cycle progression and favoring transformation (3). Furthermore, ectopic overexpression of Skp2 facilitates transformation of Rat1 cells in soft agar and in nude mouse xenograft assays (5). The oncogenic potential of Skp2 is further illustrated by developing prostate and lymphoma cancers in Skp2 transgenic mice (6, 10). However, the molecular mechanisms underlying elevated Skp2 expression in prostate cancers have not been fully explored. We and others have identified Cdh1 as the E3 ligase that promotes Skp2 destruction (2, 14). In contrast to the frequency of Skp2 overexpression, loss of Cdh1 is not a frequent event; thus it cannot explain the observation of elevated Skp2 levels in carcinomas. On the other hand, hyperactivation of the Akt pathway is considered a hallmark of many cancers including prostate cancer and it has been reported that activation of the PI3K/Akt pathway enhances p27 destruction (13). This suggests that sustained Akt activity can influence Skp2 activity. Consistent with this, studies have also demonstrated that Akt can contribute to Skp2 overexpression, although the mechanism has not been explored (1, 7). We noticed that the Skp2 protein contains a canonical Akt phosphorylation site. Thus, we hypothesize that sustained Akt activity in prostate cancer cells leads to elevated phosphorylation of Skp2, and subsequently influences Skp2 stability and its oncogenic functions. The proposal aims to determine how Akt1 phosphorylation of Skp2 disrupts its destruction mediated by the APC/Cdh1 complex and to dissect the molecular mechanisms by which Akt1 promotes Skp2 cytoplasmic localization in prostate cancer cells. Furthermore, we would like to evaluate whether specific inhibition of Akt1 can be used as a novel treatment for prostate cancer patients.

Body

Specific Aim 1: Determine how Akt1 phosphorylation of Skp2 disrupts its destruction mediated by the APC/Cdh1 complex.

Task 1: To determine whether disregulated PTEN/PI3K/Akt pathway contributes to the elevated Skp2 expression in prostate cancer (Month 1-6).

a. *To examine whether Skp2 abundance positively correlates with Akt activity, as determined by the p-Ser473 Akt signal, in a panel of prostate cancer cells including DU145 cells with normal expression of the PTEN protein, as well as LNCaP, PC3 and PC346 cells that lack the expression of PTEN (Month 1-3).*

Progress: We found that comparing with DU145 cell line with wild-type PTEN, PC3 cells that lack PTEN expression, have elevated Skp2 expression. We also found Skp2 to be overexpressed in LNCaP that is defective in PTEN (see Inuzuka et al, Cell 2012). These results suggest that loss of PTEN which activates Akt, can leads to elevated Skp2 expression.

b. *To further examine whether inhibition of PI3K/Akt activity leads to downregulation of Skp2 in PTEN-null cell lines (Month 2-3).*

Progress: As illustrated in Fig. 1, we found that inactivating the PI3K/Akt pathway by LY294002 treatment in PC3 cells leads to a severe reduction of Skp2 expression that correlates well with reduction in Akt activity.

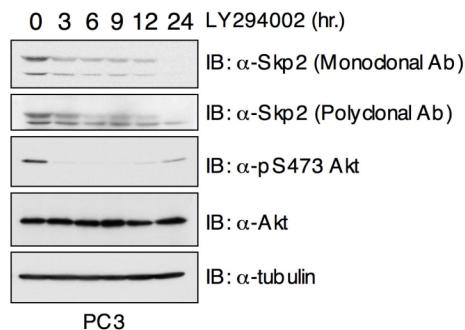


Figure 1: Inactivation of the PI3K/Akt pathway leads to downregulation of Skp2 expression levels in PC3 prostate cancer cell line with elevated Akt activity.

Immunoblot (IB) analysis of whole-cell extracts (WCE) derived from PC3 cells treated with PI3K inhibitor LY294002 for indicated time points.

c. *Milestone: To examine whether knock-down of Akt1 or Akt2 with shRNA lentiviral constructs in prostate cancer cells affects Skp2 expression (Month 3-6).*

Progress: We found that in both HeLa and SKBR3 cell lines (Fig. 2), depletion of endogenous Akt1, but not Akt2, leads to a significant decrease of Skp2 protein abundance. These results support the hypothesis that PI3K/Akt pathway governs Skp2 stability. We have also infected PC3 prostate cell line and found that similar mechanism also exists in the prostate cancer setting. Altogether, these results suggested that PI3K/Akt activity is also involved in regulating Skp2 levels in prostate cancer cells.

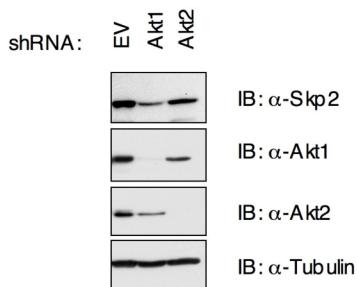


Figure 2: Depletion of Akt1, but not Akt2, results in a sharp decrease in Skp2 expression levels in SKBR3 cells.

SKBR3 cells were infected with indicated lenti-viral shRNA vectors, and then incubated with 2 ug/ml puromycin for at least four days to eliminate the non-infected cells. Cell lysates were collected and immunoblots were performed with indicated antibodies.

Task 2: To determine whether Ser72 is the major physiological Akt1 phosphorylation site (Month 6-12).

a. Immunoprecipitate endogenous Skp2 protein from PC3 cell line and perform mass spectroscopy analysis on the recovered Skp2 immunoprecipitate to analyze its phosphorylation status (month 7-8).

Progress: Using the anti-Skp2 antibody from both Santa Cruz (rabbit anti-Skp2 polyclonal antibody) and also from Zymed (mouse anti-Skp2 monoclonal antibody), we performed endogenous Skp2 IP and then run the immunoprecipitate on a SDS-gel and stained with gel-code blue reagent (from Pierce). We found that both Skp2 antibodies were not very efficient in immunoprecipitating endogenous Skp2.

We are currently in collaboration with the Cell Signaling Technology company to develop a better Skp2 antibody for immunoprecipitation. However, it will take another 10-12 months to validate the antibody. Unfortunately, due to the limitation of reagents, we cannot accomplish this specific task.

b. Alternatively, we will generate a 293T cell line stably expressing HA-Skp2 using retroviral infection, and perform HA-IP and perform mass spectroscopy analysis on the recovered HA-immunoprecipitate (month 7-9).

Progress: Additionally, we transiently transfected 293T cells with HA-Skp2 mammalian expression plasmid, and after 48 hours, the transfected 293T cells were harvested for HA-IP. After extensive washing conditions, HA-immunoprecipitates were separated on SDS-PAGE gel and then stained with gel-code blue reagent. The band containing Skp2 was excised and sent for mass spectrometry analysis. The results are shown below in Fig. 3. However, we recognize that this result cannot demonstrate whether Ser72 is a major Akt site in the prostate cancer settings, which for sure requires further investigation using a good quality Skp2 antibody.

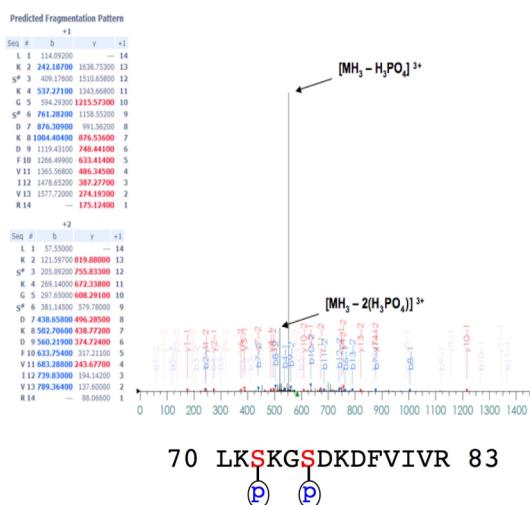


Figure 3. Detection of *in vivo* Skp2 phosphorylation status by mass spectrum analysis.
HA.Skp2 was transfected into 293T cells, then immunoprecipitated with anti-HA in the presence of phosphatase inhibitors. The immunoprecipitate was resolved by SDS-PAGE and phosphorylation was detected by mass spectrum analysis. The symbol # indicates the site of phosphorylation. Ser72 and Ser75 sites were detected to be phosphorylated *in vivo*.



c. To determine whether depletion of endogenous Akt1 and Akt2 affects Ser72 phosphorylation of Skp2 (month 9-11).

Progress: In collaboration with Cell Signaling Technology company, we are in the process of developing the p-Ser72-Skp2 antibody. As shown in Fig. 4 below, this antibody can recognize pSer72 in ectopically expressed Skp2, however, we found that this antibody cannot efficiently recognize the endogenous level of Skp2 Ser72 phosphorylation status. In this case, due to the

limitation of the reagents, we cannot further investigate whether Skp2 phosphorylation at Ser72 will be affected by depletion of Akt1 or Akt2. Cell Signaling Technology is now trying to generate more specific antibody that can detect endogenous pSer72-Skp2, which will require additional 10-12 months.

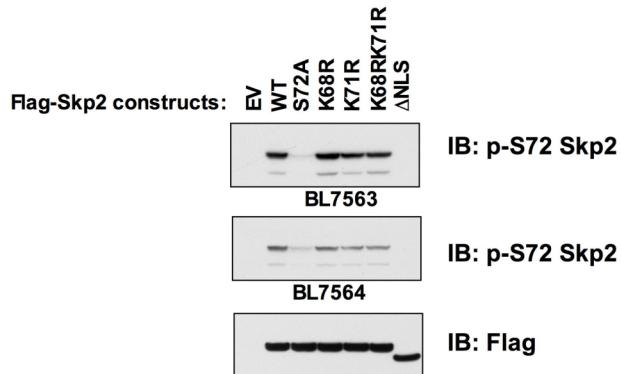


Figure 4: Development of antibody that can detect Skp2 Ser-72 phosphorylation status.

Various Flag.Skp2 constructs were transfected into 293T cells. 48 hours later, cell lysates were collected in the presence of phosphatase inhibitors. Immunoblots were performed with the indicated antibodies.

d. *Milestone: Using in vitro kinase assay and mass spectrometry assay to reveal the exact site where Skp2 protein is phosphorylated after the addition of Akt1 in vitro (month 8-10).*

Progress: We performed the *in vitro* kinase assay with active Akt1 and recombinant GST-Skp2 protein, and then sent the products for mass spectrometry analysis. However, no phosphorylation was detected. It is possibly due to the fact that the phosphorylation efficiency was too low and only a very small percentage of GST-Skp2 was phosphorylated.

To achieve this goal, we have to enhance the sensitivity of the Mass Spectrometry analysis. We have consulted Dr. John Asara in the Dept. of Medicine, BIDMC for his expert opinion. One way to enhance the sensitivity is to run the kinase reaction through a column to enrich the phospho-serine species before the mass spectrometry analysis. However, even after optimizing the experimental conditions, we did not get the expected results. Therefore, we have to rely on task 2e, which is a standard approach widely used in the kinase field to identify the exact Akt sites on Skp2.

e. We plan to generate GST-Skp2 fusion proteins whose individual potential Akt phosphorylation sites have been point-mutated which will allow us to use *in vitro* kinase assay to determine whether Ser72 is the major phosphorylation site (month 10-12).

Progress: We performed a bio-informatic scan of Skp2 protein primary sequence and found that besides the high stringency Akt site at Ser72, Skp2 contact two other low stringency Akt sites at Ser75 and Thr21. Furthermore, we also generated GST-Skp2 mutants as listed in Figure 5 and performed *in vitro* kinase assays. We found that only after mutation of the Ser72 site, the Akt-mediated Skp2 phosphorylation was severely reduced, indicating that Ser72 is the only major Akt phosphorylation site present in Skp2.

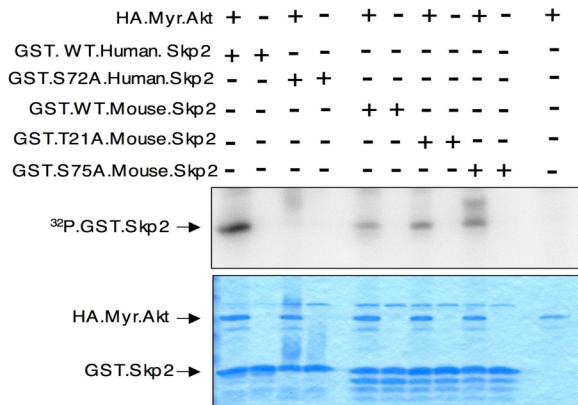


Figure 5. *In vitro* phosphorylation of the mouse Skp2 protein by Akt.

HA.Myr.Akt was transfected into 293T cells, then immunoprecipitated with anti-HA and incubated with 5 µg of indicated GST.Skp2 in the presence of γ -³²P-ATP. The kinase reaction products were resolved by SDS-PAGE and phosphorylation was detected by autoradiography.

Task 3: To determine how Akt1 phosphorylation of Skp2 at Ser72 impairs its destruction by APC/Cdh1 (Month 12-18).

a. We will use real-time RT-PCR to compare the levels of Skp2 mRNA before and after specific Akt1 depletion in the PC3 prostate cancer cells and HeLa cells (Month 12-14).

Progress: We found that in HeLa cells, inactivation of Akt1, but not Akt2 will dramatically reduce Skp2 protein abundance, and only moderately reduce Skp2 mRNA levels. These results indicate that Akt1 could influence both Skp2 protein stability and Skp2 transcription, however, Akt1 might mainly govern Skp2 activity in a post-translational mechanism. This result indicates that Akt-mediated Skp2 regulation mainly involves a post-translational mechanism.

b. We will measure the changes in Skp2 protein half-life after depletion of Akt1 in the PC3 prostate cancer cells and HeLa cells, using GFP shRNA treatment as a negative control (Month 12-14).

Progress: As illustrated in Figure 6, we found that in HeLa cells, Skp2 protein half-life is tightly controlled by the PI3K/PTEN/Akt pathway. However, due to the less-efficient siRNA transfection, we could not deplete Akt efficiently in PC3 cells. Based on our results obtained so far, in which case we observed mostly similar results in HeLa and PC3 cells regarding the role of Akt in regulating Skp2, we draw a conclusion that depletion of Akt will likely reduce Skp2 half-life in PC3 cells although further studies such as lenti-viral based-Akt depletion are required to validate this hypothesis.

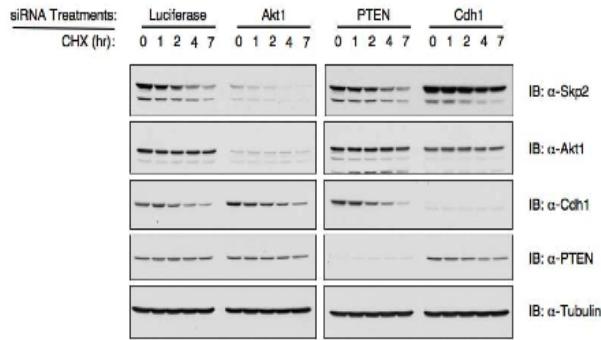


Figure 6: PI3K/Akt pathway regulates Skp2 stability in HeLa cells.

HeLa cells were transfected with the indicated siRNA oligos. After 40 hours, cells were treated with 20 ug/ml CHX. At the indicated time points, whole-cell lysates were prepared and immunoblots were performed with indicated antibodies.

c. Milestone: We will perform *Akt1*, *Cdh1* and *Akt1/Cdh1* double RNAi treatments (with mock and luciferase RNAi as negative controls) in synchronized HeLa cells, and then measure any changes in *Skp2* protein abundance and cell cycle progression. However, both *Akt1* and *Cdh1* play important roles in cell cycle regulation and *Skp2* expression levels fluctuate considerably throughout the cell cycle (Month 12-16).

Progress: We found that depletion of Akt1 leads to Skp2 downregulation while depletion of Cdh1 leads to Skp2 upregulation. More importantly, depletion of both Akt1 and Cdh1 leads to restoration of Skp2 abundance (Figure 7), indicating the Akt1 regulates Skp2 stability in a Cdh1-dependent manner.

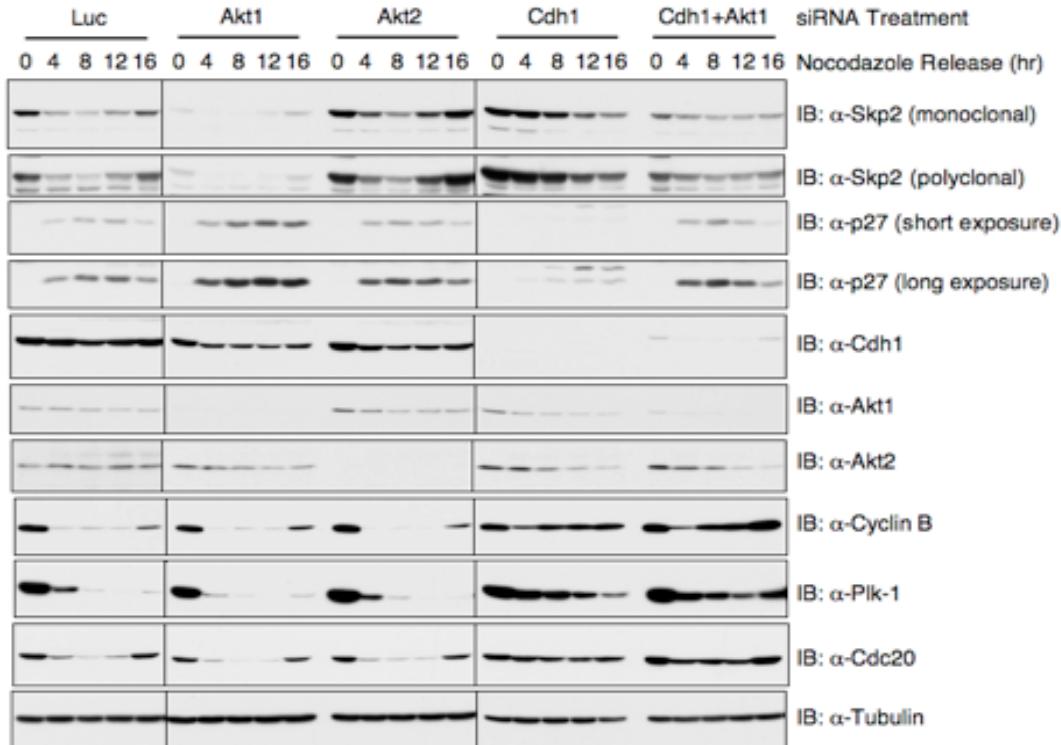


Figure 7: Phosphorylation of Skp2 by Akt1 protects Skp2 from Cdh1-mediated destruction during the cell cycle progression.

Immunoblot analysis of HeLa cells transfected with the indicated siRNA oligos, synchronized by growth in nocodazole, and then released for the indicated periods of time.

d. Milestone: To determine whether the interaction between Cdh1 and Skp2 will be disrupted by Akt phosphorylation at Ser72 (Month 15-17).

Progress: We found that in the *in vitro* GST-pull down experimental setting, mutating Ser72 and Ser75 to Asp to mimick a phosphorylation event significantly reduce Cdh1 and Skp2 interaction (Figure 8).

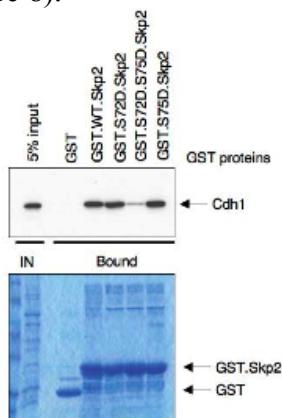


Figure 8: Phosphorylation of Skp2 by Akt1 impairs Skp2 interaction with its E3 ubiquitin ligase Cdh1. Autoradiography of 35 S-labelled Cdh1 bound to the indicated GST-Skp2 protein.

e. We will utilize an *in vivo* ubiquitination assay to demonstrate that the ability of APC/Cdh1 to promote Skp2 ubiquitination is compromised by Akt (Month 16-18).

Progress: Although we tried the *in vivo* ubiquitination assays, we have experienced strong background of non-specific ubiquitination ladders, which are frequently observed in transfection-based *in vivo* ubiquitination assays.

Specific Aim 2: Dissect the molecular mechanisms by which Akt1 promotes Skp2 cytoplasmic localization in the prostate cancer cells (Month 19-28).

Task 4: To determine the molecular mechanisms by which Akt1 promotes Skp2 cytoplasmic localization (Month 19-23).

a. Using both immunofluorescence microscopy and cellular fractionation techniques to examine whether the cellular localization of the non-phosphorylatable Skp2.S72A mutant as well as the phospho-mimetic Skp2.S72D mutant will be affected by Akt kinase activity.

Progress: As illustrated in Fig. 9, we used immunofluorescence microscopy to examine whether the cellular localization of WT-Skp2, the non-phosphorylatable Skp2.S72A mutant as well as the phospho-mimetic Skp2.S72D mutant will be affected by Akt kinase activity. Interestingly, we found that WT-Skp2 primarily localized in the nucleus and co-transfection of Myr-Akt can translocate a large portion of WT-Skp2 into the cytoplasm. However, Myr-Akt cannot promote the cytoplasmic translocation of Ser72A-Skp2, presumably due to its deficiency in Akt-mediated phosphorylation. On the other hand, a large portion of the phospho-mimetic Ser72D mutant localizes in the cytoplasm even in the absence of Myr-Akt. These studies supported the notion that Akt could promote the cytoplasmic localization of Skp2 mainly by phosphorylating Skp2 at the Ser72 site.

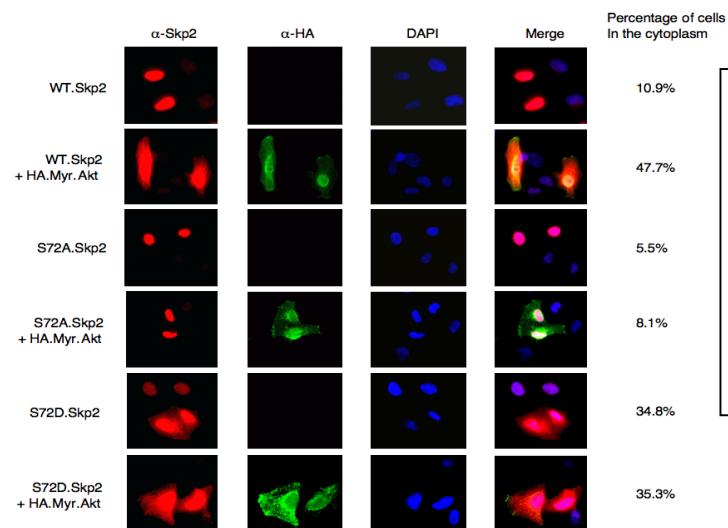


Figure 9: Phosphorylation of Skp2 by Akt1 promotes the cytoplasmic translocation of Skp2 in a Ser72-dependent manner.

Immunofluorescence results to examine the effects of Akt1 on the cytoplasmic localization of the various indicated Skp2 constructs.

b. To investigate whether expression of HA-Myr-Akt (a constitutively active form of Akt1) resulted in an enhanced association between 14-3-3 and Skp2 (Month 19-23).

Progress: As illustrated in Fig. 10 below, we found that Skp2 interacts with endogenous 14-3-3 and furthermore, ectopic expression of Myr-Akt could moderately increase the interaction between Skp2 and endogenous 14-3-3.

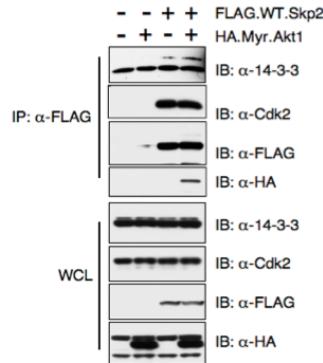


Figure 10: Phosphorylation of Skp2 by Akt1 triggers Skp2 interaction with 14-3-3.

293T cells were transfected with the indicated plasmids. 30 hours post-transfection, cells were harvested for anti-Flag immunoprecipitation analysis. Both whole cell lysates and the immunoprecipitates were subjected to immunoblot analysis with the indicated antibodies.

c. Milestone: We plan to investigate whether phosphorylation of Skp2 by Akt1 disrupts the interaction between the importin complex and Skp2 (Month 21-23).

Progress: As illustrated in Fig. 11 below, we found that phospho-mimetic Skp2 failed to interact with importin $\alpha 5$, indicating that phosphorylation of Skp2 by Akt1 disrupts the interaction between the importin complex and Skp2.

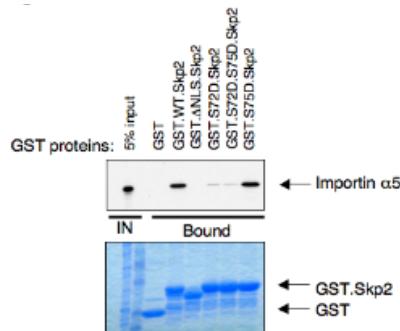


Figure 11: Phosphorylation of Skp2 by Akt1 disrupts the interaction between Skp2 and the importin complex substrate recruiting unit.

Autoradiography of ^{35}S -labelled importin $\alpha 5$ bound to the indicated GST-Skp2 protein.

Task 5: Determine whether elevated Akt activity is causative for cytoplasmic Skp2 staining in aggressive prostate cancers (Month 24-28).

a. We will investigate whether there is increased cytoplasmic Skp2 localization in the prostate cancer cell lines PC3 and LNCaP with elevated Akt activities due to loss of PTEN (Month 24-26).

Progress: We found that in multiple cell lines including, HeLa, MDA-468 and SKBR3 cancer cell lines, elevated Akt promotes Skp2 cytoplasmic localization.

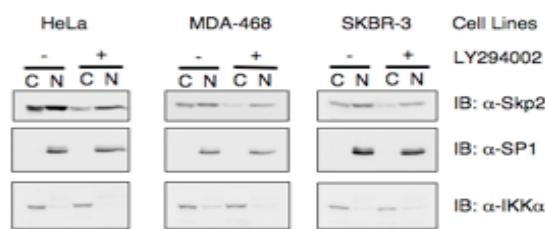


Figure 12: Phosphorylation of Skp2 by Akt1 promotes cytoplasmic localization.

Immunoblot analysis of Nuclear (N) and Cytoplasmic (C) fractions of HeLa, MDA-MB468 and SKBR3 cells treated with LY294002 (or DMSO as a negative control) for 12 hours.

b. Furthermore, we will investigate whether there is enhanced Skp2 nuclear import after specific depletion of Akt1, but not Akt2, in these cells. This experiment will allow us to determine whether Akt1 activity is causative for the observed Skp2 cytoplasmic translocation (Month 25-27).

Progress: To directly assess the role of Akt in Skp2 cytoplasmic localization, we transfected the WT, as well as S72A (phospho-deficient) and S72D.S75D (phospho-mimetic) Skp2 in the presence or absence of active Akt. Interestingly, we found that active form of Akt can promote WT, but not S72A-Skp2 cytoplasmic localization (Figure 13). On the other hand, the phospho-mimetic, S72D.S75D-Skp2 constantly localizes in the cytoplasm (Figure 13), further supporting the notion that Akt-dependent phosphorylation of Skp2 promotes its cytoplasmic localization.

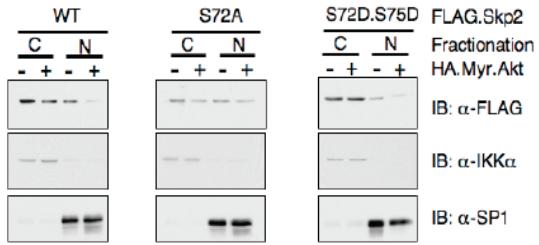


Figure 13: Phosphorylation of Skp2 by Akt1 promotes Skp2 cytoplasmic localization.
Immunoblot analysis of Nuclear (N) and Cytoplasmic (C) fractions of HeLa cells transfected with the indicated plasmids.

c. Milestone: With the assistance from my collaborator Dr. Pier Paolo Pandolfi and pathologist Dr. Stuart Schnitt, I have access to a large set of prostate cancer tissue samples. We will further carry out this research using immunohistochemistry in these clinical samples, to investigate whether a positive correlation exists between elevated Akt activity and increased cytoplasmic Skp2 staining (Month 25-28).

Progress: Currently there is no commercially available excellent quality of anti-Skp2 antibody that is suitable for IHC. In collaboration with Cell Signaling Technology, we are in the process of developing IHC-Skp2 antibody so that we can perform immunohistochemistry on a large set of prostate cancer tissue samples to examine whether there is a correlation between elevated Akt (as evidenced by pSer473-Akt signal) and increased cytoplasmic localization of Skp2. Hence, the successful accomplishment of this specific task is limited by reagents as well.

Specific Aim 3: Evaluate whether specific inhibition of Akt1 can be used as a novel anti-prostate cancer solution (Month 29-36).

Task 6: Dissect the molecular mechanisms of the Akt isoform specificity towards Skp2 phosphorylation in prostate cancers (Month 29-32).

a. We plan to perform endogenous Skp2 immunoprecipitation and then examine the Skp2 interaction with endogenous Akt1 versus Akt2 (Month 29-31).

Progress: As illustrated in Figure 14, we were able to demonstrate that in multiple cancer cell lines including U2OS and SKBR3, depletion of Akt1, but not Akt2 led to a severe decrease in Skp2 expression. This result highly suggests that Akt1, but not Akt2, specifically regulates Skp2.

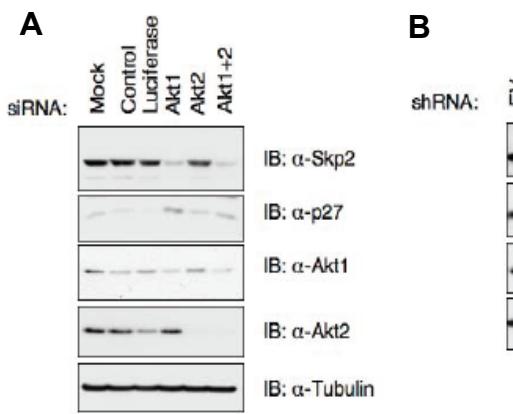


Figure 14: Depletion of Akt1 but not Akt2 led to decreased Skp2 expression.

Immunoblot analysis of whole cell lysates derived from U2OS (A) or SKBR3 (B) cancer cell lines transfected with the indicated siRNA oligos.

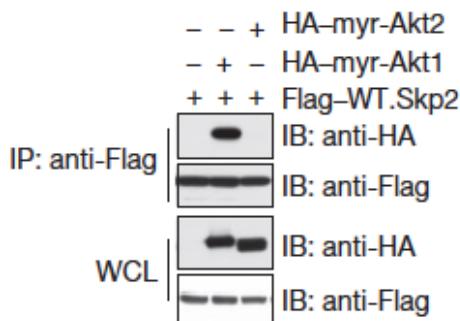


Figure 15: Akt1, but not Akt2, specifically interacts with Skp2 *in vivo*.

Immunoblot analysis of whole cell lysates and immunoprecipitations derived from HeLa cells transfected with the indicated plasmids

b. As an alternative approach, we plan to ectopically overexpress HA-Skp2 constructs in the PC3 cells. Following HA-immunoprecipitation, we will examine the interaction between the immunoprecipitated Skp2 and endogenous Akt1 and Akt2 (Month 29-31).

Progress: Consistent with the notion that Akt1 specifically regulates Skp2, under ectopic expression experimental conditions, we were able to detect interaction between Skp2 and Akt1, but not with Akt2 (Figure 15). However, we recognize that further studies are required to further understand the underlying molecular mechanism.

c. Milestone: It is plausible that the nuclear localization of Akt1 which has been observed in some cell lines may allow it to interact with nuclear Skp2 and promote nuclear export, and that in contrast, the predominantly cytoplasmic localization of Akt2 may restrict its accessibility to Skp2. To address this question, we plan to attach a Nuclear Export Signal (NES) to Skp2, creating a mutant Skp2 that primarily localized in the cytoplasm. Then we will continue to examine the ability of this mutant Skp2 to interact with Akt1 versus Akt2 (Month 30-32).

Progress: We have carefully discussed this experimental design with our collaborator, Dr. Alex Toker. Dr. Toker made us aware of the fact that the cellular localization of Akt1 versus Akt2 is still controversial. Therefore, we modified our specific task to the question of exploring the additional molecular mechanisms responsible for Skp2 cytoplasmic localization. This is in part due to the fact that the Ser72 site in human Skp2 is not well conserved in the mouse Skp2 sequence, indicating another yet unidentified mechanisms controlling Skp2 cytoplasmic localization, which is frequently observed in more aggressive form of breast and prostate human cancers. As illustrated in Figure 16, we found that p300 could acetylate Skp2 at the K68 and K71 sites. More importantly, unlike Ser72, the K68/K71 sites are evolutionally conserved (Figure 17) and we further demonstrated that as these sites are localized in the NLS sequence, acetylation of Skp2 promotes cytoplasmic localization of Skp2 (Figure 18) similarly as Ser72 phosphorylation does. Most importantly, we also showed that Akt could promote p300-dependent acetylation of

Skp2 (Figure 19). This indicates that in human cells, Akt could presumably regulate Skp2 in two independent pathways, either by directly phosphorylating Skp2 at Ser72, or by promoting the p300-dependent acetylation of Skp2. On the other hand, in mouse where the Skp2 Ser72 site is not conserved, Akt mainly regulates Skp2 by an acetylation-dependent mechanism (Figure 20).

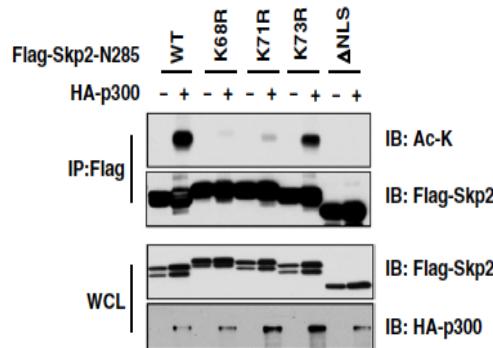


Figure 16: Skp2 is acetylated in vivo at K68/K71.

Immunoblot analysis of whole cell lysates and immunoprecipitates derived from HeLa cells transfected with the indicated plasmids

	68	71	73	77			
Human (63-80)	ES	PPR	KRL	KS	KGS	DKD	FV
Chimpanzee (63-80)	ES	PPR	KRL	KS	KGS	DKD	FV
Macaque (63-80)	ES	PPR	KRL	KS	KG	SEK	DV
Dog (63-80)	QS	PPR	KRL	KS	KGN	NDK	DV
Cow (63-80)	QS	PPR	KRL	KS	KGN	NDK	DV
Horse (63-80)	QS	PPR	KRL	KS	KGN	NDK	DV
Pig (63-80)	QS	PPR	KRL	KS	KGN	EKD	DV
Rat (63-80)	QS	PPR	KRL	KS	KGS	DKD	FV
Mouse (63-80)	QS	PPR	KRV	KGKG	SDK	DV	

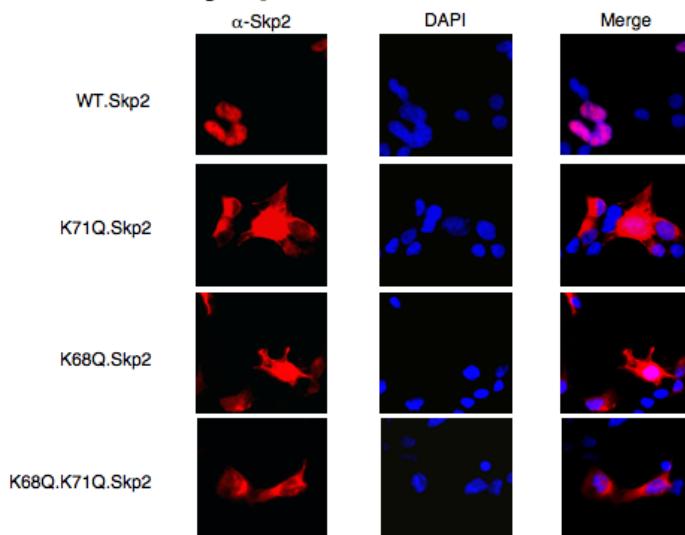


Figure 17: Alignment of the Skp2 acetylation sites across different species.

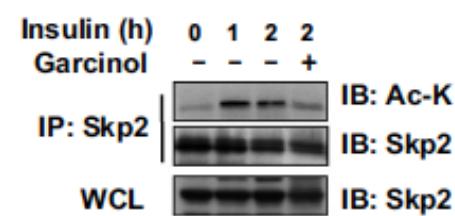


Figure 19: Akt promotes acetylation of Skp2 by activating p300.

Immunoblot analysis of whole cell lysates and anti-Skp2 immunoprecipitates derived from HeLa cells treated with Insulin. Where indicated, p300 inhibitor, Garcinol was added.

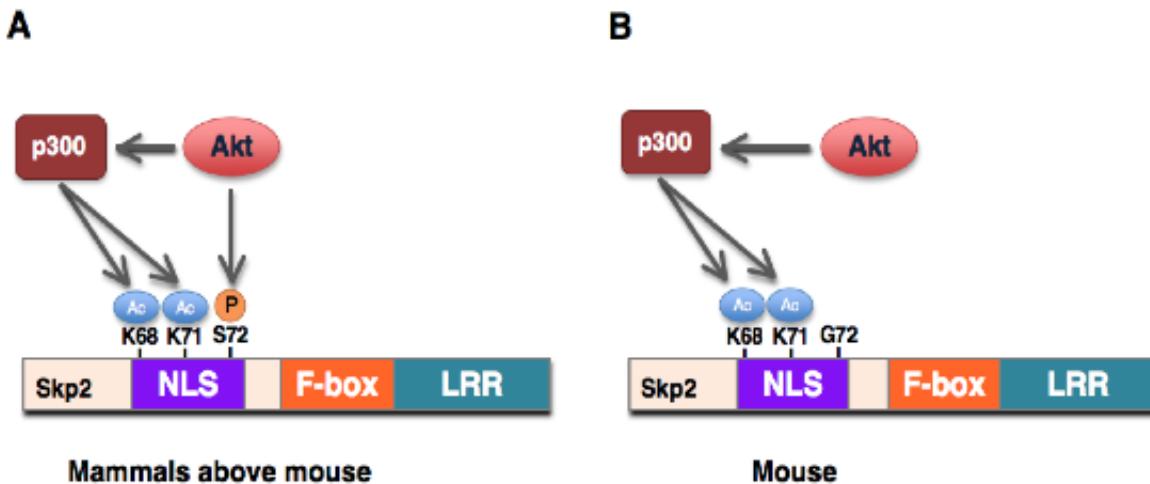


Figure 20: Proposed model for how Skp2 oncogenic functions are governed by both Akt and p300 signaling pathways

Task 7: Determine whether specific inhibition of Akt1, but not Akt2, leads to cell cycle arrest or cellular death in prostate cancer cells (Month 33-36).

a. We will infect the PC3 and LNCaP prostate cancer cells with a lentiviral shRNA vector against Akt1 or Akt2 (with GFP shRNA as a negative control). After drug selection, the resulting clones will be analyzed by Western blots for the efficiency of shRNA knockdown as well as the effects of Akt1 depletion on the Skp2 downstream targets such as p27 and FOXO1; and by FACS analysis for the cell cycle profiles (Month 33-25).

Progress: Unfortunately, depletion of Akt1 in LNCaP cells led to severe cell death, indicating that LNCaP cells are addicted to the Akt oncoprotein, a phenotype described as “oncogene addition”. Alternatively, we transfected LNCaP cells with either wild-type or active form (acetylation-mimetic, KLKL mutant form) of Skp2. As illustrated in Figure 21, we found that the Acetylation-mimetic Skp2 mutant exerts elevated oncogenic abilities by promoting the destruction of the FOXO1 tumor suppressor. Consistent with this notion, we found that cells expressing KLKL-Skp2 exhibited elevated BrdU labeling index, indicating elevated entry into the S phase (Figure 22).

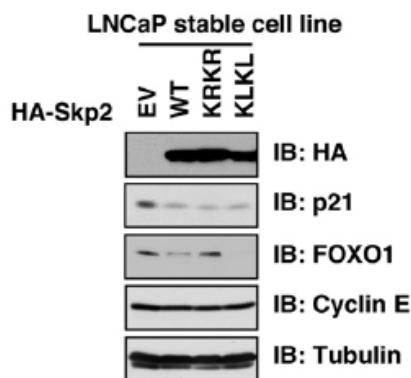


Figure 21: Acetylation-mimetic Skp2 mutant exerts elevated oncogenic abilities by promoting the destruction of FOXO1 tumor suppressor.
Immunoblot analysis of whole cell lysates derived from LNCaP cells transfected with the indicated Skp2 constructs.

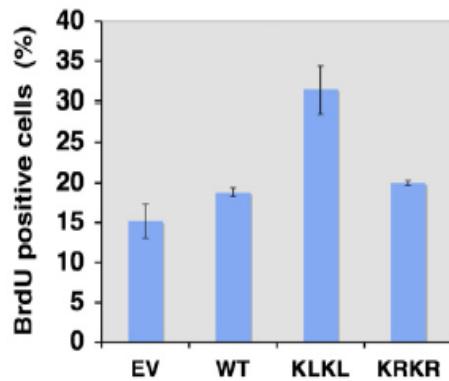


Figure 22: Acetylation-mimetic Skp2 mutant exerts elevated oncogenic abilities by promoting the destruction of FOXO1 tumor suppressor to accelerate S phase entry.

BrdU labeling assays to detect S phase entry differences among LNCaP cells transfected with the indicated Skp2 constructs.

b. Milestone: To address the importance of Skp2 in this process, lentiviral shRNA against Skp2 will also be included in this assay, to compare whether specific knockdown of Skp2 results in a similar phenotype as depletion of Akt1 (Month 33-35).

Progress: First of all, we found that Skp2^{-/-} MEFs migrated much less than WT-MEFs (Figure 23), indicating that Skp2 might promote cellular migration but the underlying mechanism is unknown. Depletion of Skp2 in the DU145 prostate cancer cell line led to elevated p27 expression that could lead to reduced cell growth (Figure 24). More importantly, we found that loss of Skp2 in DU145 cell lines leads to a significant increase in the expression of the E-cadherin tumor suppressor (Figure 24). E-cadherin has been shown previously to block cell migration and loss of E-cadherin is associated with more aggressive form of prostate cancer and metastasis. This result also indicates that E-cadherin might be a Skp2 downstream ubiquitin substrate.

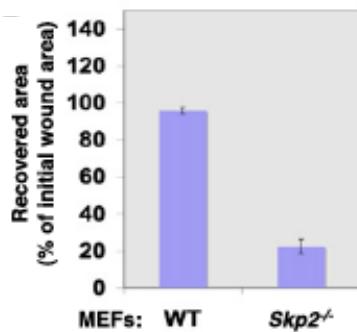
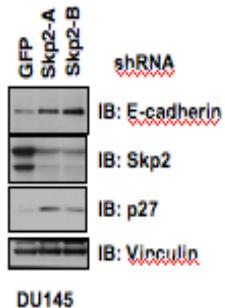


Figure 23: Depletion of endogenous Skp2 leads to retarded cell migration.

Transwell assays to measure cell migration ability between WT and Skp2^{-/-} MEFs.

Figure 24: Depletion of endogenous Skp2 in prostate cancer cell lines leads to growth arrest and reduced cell migration.
Immunoblot analysis of whole cell lysates derived from DU145 prostate cancer cell lines infected with the indicated shRNA lentiviral constructs.



c. Furthermore, a destruction-box deleted, constitutively stable version of Skp2 will be overexpressed in the resulting Akt1-depleted cells to assess whether overexpression of Skp2 will rescue the cells from either cell cycle arrest or cellular death (Month 34-36).

Progress: As our results indicates that Acetylated Skp2 is defective in binding to Cdh1, thereby behaving like D-box-deleted version of Skp2, in the following up studies, we are using KLKL-Skp2 as a non-degradable version of Skp2 (Figure 25). Interestingly, we found that compared to WT or KRKR (acetylation-deficient version of Skp2), expression of KLKL-Skp2 (acetylation-mimetic version) greatly elevated cell migration (Figure 26). We further demonstrated that expression of KLKL-Skp2 in DU145 prostate cancer cell line could lead to rapid degradation of E-cadherin, which in part may explain its oncogenic ability in promoting cell migration (Figure 27).

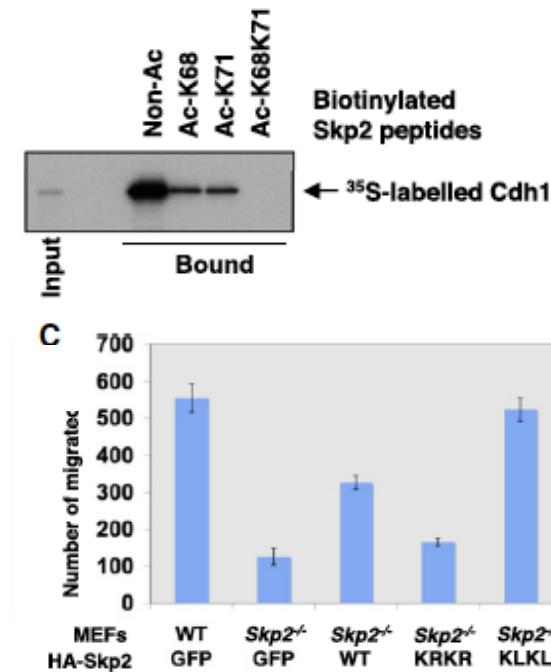


Figure 25: Acetylated Skp2 is defective in binding to Cdh1, thereby behaving like D-box-deleted version of Skp2.

Autoradiography of ^{35}S -labeled Cdh1 bound to the indicated biotinylated peptides.

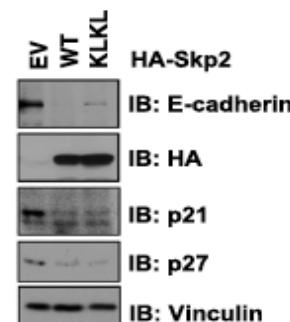


Figure 27: Acetylated Skp2 promotes cell migration by degrading E-cadherin.

Immunoblot analysis of whole cell lysates derived from DU145 prostate cancer cell lines infected with the indicated lentiviral constructs.

d. It is highly plausible that we will be unable to establish any stable cell line with permanently low Skp2 and Akt1 expression. To overcome this problem, we will use the tetracycline-inducible shRNA system. In this experimental system, in the generated stable cell lines, there is no depletion of Akt1 or Skp2 until the addition of tetracycline. This will be an optimal approach to address the contribution of Skp2 in Akt1 inactivation-induced cell cycle arrest or apoptosis (Month 34-36).

Progress: Unfortunately, we experienced a great degree of leakiness when using the Tet-inducible system. Therefore, we decided not to further pursue this research direction as it is not suitable for our original experimental design and could potentially lead to artificial results that complicate the explanation of the experimental results.

e. Milestone: We will also use the normal pharmaceutical agents typically used in cancer treatment including the pan-Akt and the PI3K inhibitors in these assays to examine whether inhibition of Akt can be used to inhibit tumor cell proliferation (Month 34-36).

Progress: As illustrated in Figure 28, PI3K inhibitor treatment caused a significant reduction in Skp2 expression and subsequent upregulation of the CDK inhibitor, p27, in multiple cancer cell lines. As p27 expression has been well characterized to cause G1 arrest, we anticipate that this will lead to growth arrest in the G1 cell cycle phase, presumably due to reduced CDK1/2 kinase activities. Furthermore, as shown in Figure 14, we also found that depletion of Akt1 in multiple cancer cell lines could also lead to downregulation of Skp2 and upregulation of p27. This result indicates that behaving similarly to PI3K inhibitors, specific Akt inhibitors could also lead to growth arrest in part due to elevated p27 expression. In collaboration with Dr. Alex Toker, we have examined several Akt isoform inhibitors. However, none of them could exert Akt1 or Akt2-specific inhibition. Therefore, due to technical difficulties, we could not accomplish the specific task proposed in this sub-aim.

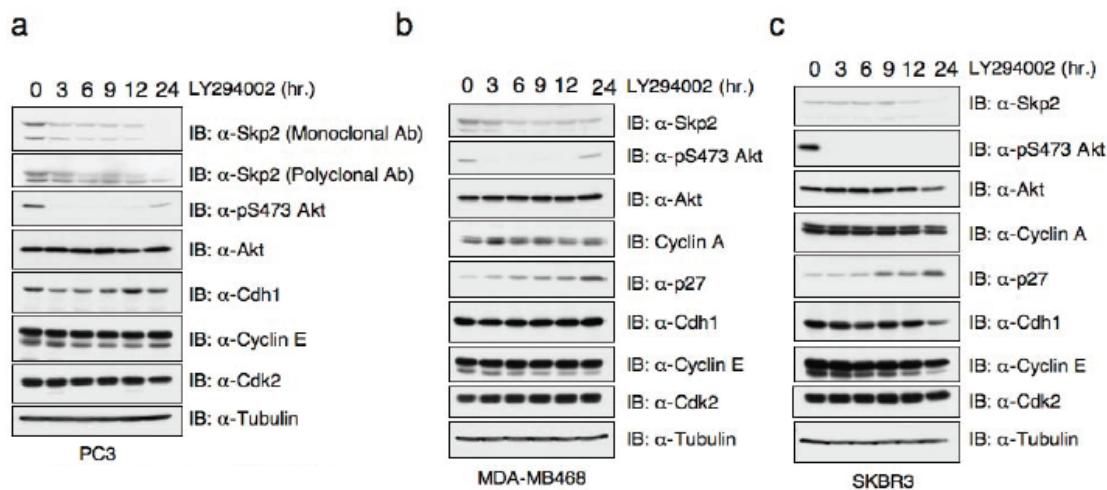


Figure 28: PI3K inhibitor caused reduced Skp2 expression and upregulation of p27 in multiple cancer cell lines.

Immunoblot analysis of whole cell lysates derived from PC3 prostate cancer cell line (A), MDA-MB468 (B) and SKBR3 (C) breast cancer cell lines treated with the PI3K inhibitor, LY294002 for the indicated time of periods.

Key Research Accomplishments:

- We found that Skp2 is phosphorylated by Akt1 at Ser72.
- We also showed that inactivation of PI3K/Akt pathway in PC3 prostate cancer cell line leads to downregulation of Skp2 protein abundance.
- We further showed that Akt1-mediated phosphorylation of Skp2 leads to stabilization of Skp2. This finding offers a novel explanation for the frequent Skp2 overexpression in prostate cancers.
- We further illustrated that phosphorylation of Skp2 by Akt impairs Skp2/Cdh1 interaction, thus allowing Skp2 to escape Cdh1-mediated destruction.
- We also generated an antibody that can recognize the p-Ser72 form of Skp2, which will be very useful reagent to further explore how Akt1 regulate Skp2 phosphorylation to affect Skp2 oncogenic activity.
- We found that phosphorylating Skp2 at Ser72 by Akt promotes its cytoplamic localization.
- We further showed this is largely due to the fact that Akt-mediated phosphorylation of Skp2 impairs its interaction with the importin complex.
- In addition, Akt could moderately trigger Skp2 interaction with 14-3-3.
- We defined that Akt could activate p300 to promote Skp2 acetylation.
- Acetylation of Skp2 stabilizes Skp2 by impairing its interaction with Cdh1.
- Acetylated Skp2 exhibits elevated oncogenic ability in part by promoting E-cadherin degradation.
- Acetylated Skp2 is translocated to the cytoplasm.
- Specific inhibition of Akt and PI3K in multiple cancer cell lines lead to reduced Skp2 expression, which subsequently lead to elevated p27 expression to suppress cell cycle.

Reportable Outcomes:

We published four relevant papers as listed below:

1. Inuzuka, H., Shaik, S., Onoyama, I., Tseng, A., Gao, D., Maser, R., Zhai, B., Wan, L., Gurierrez, A., Lau, A., Xiao, Y., Christie, A., Aster, J., Settleman, J., Gygi, S., Kung, A. L., Look, T., Nakayama, K. I., DePinho, R. A. and Wei, W. (2011) SCFFbw7 regulates cellular apoptosis by targeting the Mcl-1 oncoprotein for ubiquitination and destruction. *Nature* 471:104-9.
2. Wang, Z., Gao, D., Fukushima, H., Inuzuka, H., Liu, P., Wan, L., Sarkar, F. H., and Wei, W. (2011) Skp2: a novel potential therapeutic target for prostate cancer. *BBA Reviews on Cancer* 1825(1): 11-17
3. Gao, D.*, Inuzuka, H.*, Tan, M.* , Fukushima, H., Locasale, J. W., Liu, P., Zhai, B., Wan, L., Shaik, S., Lyssiotis, C.A., Gygi, S. P., Toker, A., Cantley, L. C., Asara, J. M., Harper, J. W.# and Wei, W.# (co-corresponding author) (2011) mTOR drives its own activation via SCF^{b-TRCP}-dependent degradation of the mTOR inhibitor DEPTOR. *Molecular Cell* 44(2): 290-303
4. Wan, L., Zou, W., Gao, D., Inuzuka, H., Fukushima, H., Berg, A. H., Drapp, R., Shaik, S., Hu, D., Lester, C., Eguren, M., Malumbres, M., Glimcher, L. H. and Wei, W. (2011) Cdh1 Regulates Osteoblast function through an APC/C-independent modulation of Smurf1. *Molecular Cell* 44(5): 721-33
5. Wang, Z., Fukushima, H., Gao, D., Inuzuka, H., Wan, L., Lau, A. W., Liu, P. and Wei, W. (2011) The two faces of Fbw7 in cancer drug resistance. *BioEssays* 33(11): 851-9
6. Wang, Z., Inuzuka, H., Fukushima, H., Wan, L., Gao, D., Shaik, S., Sarkar, F. H., and Wei, W. (2011) The emerging function of the Fbw7 tumor suppressor in stem cell differentiation. *EMBO Report* 13:36-43.
7. Wang, G., Wang, Z., Sarkar, F. H., and Wei, W. (2012) Targeting prostate cancer stem cells for cancer therapy. *Discovery Medicine* 13: 135-42
8. Shaik, S., Nucera, C., Inuzuka, H., Gao, D., Garnaas, M., Frechette, G., Harris, L., Wan, L., Fukushima, H., Husain, A., Nose, V., Fadda, G., Sadow, P. M., Goessling, W., North, T., Lawler, J. and Wei, W. (2012) SCF^{b-TRCP} suppresses angiogenesis and thyroid cancer cell migration by promoting ubiquitination and destruction of VEGF-Receptor-2. *Journal of Experimental Medicine* 209(7): 1289-1307
9. Inuzuka, H.* , Gao, D.* , Finley, L., Yang, W., Wan, L., Fukushima, H., Chin, Y. C., Zhai, B., Shaik, S., Lau, A. W., Wang, Z., Gygi, S. P., Nakayama, K., Teruya-Feldstein, J., Toker, A., Haigis, M., Pandolfi, P.P. and Wei, W. (2012) Acetylation-Dependent Regulation of Skp2 Function. *Cell* 150(1): 179-193

Conclusions: We showed that Akt1, but not Akt2, directly controls Skp2 stability by a mechanism that involves degradation by the APC/Cdh1 ubiquitin ligase complex. Furthermore, we showed that in Prostate Cancer cell line PC3, inactivation of PI3K/Akt leads to downregulation of Skp2 protein levels. This is partially due to the fact that phosphorylation of Skp2 by Akt impairs Skp2 interaction with its E3 ligase Cdh1, allowing Skp2 to escape Cdh1-mediated proteolysis. In addition, we found that Ser72 is localized within a putative Nuclear Localization Sequence (NLS) and thus we also propose that phosphorylation of Ser72 by Akt might lead to Skp2 cytoplasmic translocation.

We believe that our proposed studies will provide a novel mechanism to explain the observations of Skp2 overexpression in prostate cancers. They will also expand our knowledge of how specific kinase signaling cascades influence proteolysis governed by APC/Cdh1 complexes, and will provide further evidence that elevated Akt activity is responsible for the cytoplasmic Skp2 staining which is observed in more aggressive and advanced cases of prostate cancer. These studies should allow us to assess whether inhibition of the PI3K/Akt pathway could be a novel and therapeutically promising way to block the growth of prostate tumor cells by downregulation of Skp2. Furthermore, our work will provide the rationale for developing Akt1-specific inhibitors as efficient anti-cancer drugs for prostate cancer patients.

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